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Enzyme-Linked Immunosorbent Assay Detects a Potential Soluble Form of the Erythropoietin Receptor in Human Plasma

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The erythropoietin receptor (EpoR) is a type I transmembrane protein that is a member of the family of hemopoietin receptors. Several members of this family have soluble receptor forms that are secreted by the cells rather than expressed on the cell surface. An alternatively spliced EpoR transcript has been described in human erythroid precursors that, if translated, would produce a truncated, soluble EpoR lacking the transmembrane domain. To determine if the human EpoR is expressed in a soluble form, we developed a sensitive enzyme-linked immunosorbent assay (ELISA) for the EpoR, and we analyzed human serum and plasma. Sheep were immunized with a fusion protein (EREx) consisting of glutathione-S-transferase (GST) and the human EpoR extracellular domain. The sheep antiserum was affinity-purified on immobilized EREx, and then used in a two-stage antigen capture ELISA. The plasma from 20 normal subjects was studied with this assay. There was wide variability in the levels of soluble EpoR in these subjects (range, <10–2,200 ng/ml). An average value of 550 ± 735 ng/ml for soluble EpoR was obtained in these normals. Protein A adsorption of the test plasma prior to the assay had no effect on the values obtained. Assay of serum from the same normal subjects showed an average decrease of 88% in soluble EpoR levels compared to plasma. There was no correlation between hematocrit and soluble EpoR level. This assay may have utility in the further elucidation of erythropoietin physiology. © 1996 Wiley-Liss, Inc.

Key words: erythropoietin receptor, erythropoiesis, cytokine receptors

INTRODUCTION

Erythropoietin (Epo) is the primary hormonal mediator of terminal erythropoiesis [1]. Epo supports erythroid differentiation by preventing apoptosis in maturing erythroid cells. Both murine and human EpoR have been cloned and are members of the large family of hemopoietin receptors [2]. This family includes the receptors for GM-CSF, G-CSF, IL-2, IL-3, IL-6, growth hormone, and many others. Most of the receptors in this family have a multisubunit structure. In addition, many of these receptors have soluble forms detectable in human blood, e.g., the IL-4 receptor [3] and the IL-6 receptor [4]. The physiologic role of soluble hematopoietic growth factor receptors is unclear and is an area of active investigation [5].

The EpoR gene codes for a 66-kDa type I transmembrane protein which is posttranslationally modified to 78 kDa in its fully processed, cell surface form [6]. The EpoR is the primary Epo binding protein on erythroid cells [7] and may form a ligand-dependent homodimer [8,9,10]. Todokoro et al. [11] have detected a potential soluble form of EpoR during reverse transcriptase polymerase chain reaction amplification of mRNA from human erythroid progenitor cells. Their data show that about 20% of the amplified cDNA predicts a truncated EpoR protein lacking both a transmembrane domain and a cytoplasmic domain. This alternatively spliced form results from an alternative acceptor site 5′ to exon 5. This results in a shift in translational reading frame that leads to early chain termination. Nakamura et al. [12] have reported similar results.

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In order to determine if this alternatively spliced mRNA produces a soluble form of human EpoR protein, we developed an enzyme-linked immunosorbent assay (ELISA) for the human EpoR extracellular domain. We report here on the use of this assay in measuring the potential soluble EpoR in plasma.

MATERIALS AND METHODS

Materials

Affigel 15 was obtained from Bio-Rad (Hercules, CA). Sephacryl S-300 and glutathione agarose were from Pharmacia (Piscataway, NJ). N-hydroxysuccinimide long-chain biotin, protein G agarose, protein A agarose, 3,3′,5,5′-tetramethylbenzidine, dihydrochloride (TMB) substrate, avidin coupled to horseradish peroxidase (HRP), and rabbit anti-sheep IgG coupled to HRP were from Pierce (Rockford, IL). Enzyme-linked chemiluminescence reagent was from Amersham (Arlington Heights, IL). ELISA-grade bovine serum albumin was from Sigma (St. Louis, MO). All other reagents were obtained from standard sources and were of high quality.

Preparation of Recombinant Human EpoR Extracellular Domain Fusion Protein

The cloning, purification, and characterization of the recombinant human EpoR extracellular domain has been previously reported [7]. Briefly, polymerase chain reaction-amplified cDNA representing the entire extracellular domain of human EpoR was ligated into the bacterial expression vector pGEX (Pharmacia). After transformation of *Escherichia coli* and subsequent isopropyl b-D-thiogalactopyranoside (IPTG) induction, a fusion protein was expressed with an N terminal half which is glutathione-S-transferase (GST), and a C terminal half which is the EpoR extracellular domain. This fusion protein is called EREx. The bulk of the expressed protein is contained in insoluble bacterial inclusion bodies and is not functionally active. As previously described [7], a small fraction of the EREx is soluble and, after affinity chromatography on Epo agarose, binds ^{125}I-Epo with nanomolar affinity.

In order to obtain enough material to immunize animals, EREx was purified from the bacterial inclusion bodies. Growth of bacteria, IPTG induction of EREx expression, and preparation of bacterial lysates were as previously described [7]. A typical preparation used 1 l of bacterial culture. The pellet remaining after centrifugation of the bacterial lysate at 15,000*g* for 15 min contained the inclusion bodies. This pellet was washed by resuspension in 9 volumes of 100 mM NaCl, 50 mM Tris, pH 8.0, 0.5% Triton X-100, and 10 mM ethylenediaminetetraacetic acid (EDTA), followed by centrifugation at 15,000*g* for 15 min. The wash and centrifugation were repeated two more times. The pellet remaining after these washes was resuspended in 3 ml of denaturing buffer (6 M urea, 50 mM Tris, pH 8.0, 0.5 M NaCl). The sample was boiled for 5 min in 5% 2-mercaptoethanol and then chromatographed at 4°C on a 2.5 × 120 cm column of Sephacryl S-300 equilibrated in denaturing buffer. Seven-ml fractions were collected. The peak containing the EREx fusion protein was identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), pooled, and dialyzed against 150 mM NaCl, 20 mM Tris, pH 7.5. The final purified protein was >95% homogeneous by SDS PAGE.

GST was obtained in an analogous manner. *E. coli* transformed with the unmodified pGEX vector were induced with IPTG, and the lysate containing the expressed GST was chromatographed on glutathione agarose as described by the manufacturer (Pharmacia, Piscataway, NJ).

Preparation of Sheep Anti-Human EpoR Antibody

Sheep were immunized with EREx or GST for production of specific polyclonal antiserum (Greystone Therapeutics, Hardwick, MA). Preimmune serum was also obtained. Affinity-purified polyclonal antibodies were obtained by chromatography of the sheep serum on EREx or GST agarose. EREx or GST agarose was obtained by coupling the proteins to Affigel 15, as described by the manufacturer (Bio-Rad). Affinity purification on these resins was performed using standard methods [13]. Preimmune sheep IgG was obtained by chromatography of preimmune serum on protein G agarose as described by the manufacturer (Pierce).

Since EREx is a fusion protein that includes GST, anti-EREx antibodies contain significant amounts of anti-GST. Affinity-purified anti-EREx was depleted of anti-GST antibodies by extensive adsorption on GST agarose. This procedure completely eliminated anti-GST antibodies, as assessed by the inability of the antibodies to identify purified GST on an immunoblot (data not shown). All anti-EREx antibodies used in the present study were affinity-purified and then anti-GST-depleted in this way. For some experiments, the affinity-purified anti-EREx was adsorbed with EREx agarose for 1 hr at 37°C prior to use in the ELISA, in order to demonstrate specificity.

Following anti-GST depletion, the anti-EREx antibodies (100 μg/ml) were biotinylated with N-hydroxysuccinimide long-chain biotin as described by the manufacturer (Pierce).

The affinity-purified anti-EREx blocks binding of ^{125}I-Epo to EpoR-bearing cell lines [7]. The anti-EREx antibody identifies the EpoR but not the IL-3 or GM-CSF receptors on immunoblots of cell lines (data not shown).

Enzyme-Linked Immunosorbent Assay for Soluble Human EpoR

One hundred and fifty μl of anti-EREx (40 μg/ml) in 100 mM NaCl, 50 mM phosphate, pH 7.5 (PBS), were

added to each well of a 96-well ELISA plate and allowed to bind for 2 hr at 37°C. The plates were washed twice in PBS and then incubated with 3% bovine serum albumin (BSA) in PBS for 2 hr at 37°C. Plates were washed twice in PBS, and then 150 μl of the sample to be tested were added. Serum or plasma was either tested undiluted or diluted as indicated with PBS. Plates were incubated 2 hr at 37°C and then washed four times with PBS. One hundred and fifty μl of biotinylated anti-EREx (0.4 μg/ml in PBS containing 3% BSA) were then added, and the plates were incubated for 2 hr at 37°C. The plates were washed four times with PBS, followed by addition of 150 μl of avidin-HRP (1:10,000 dilution of commercial stock in PBS containing 3% BSA). After incubation for 1 hr at 37°C, the plates were washed four times. One hundred and fifty μl of TMB substrate were added. After 20 min at room temperature, the absorbance at 450 nm was determined with a Bio-Rad microplate reader. A standard curve constructed with purified EREx (Fig. 1) was performed for each plate. Human plasma samples that had an absorbance beyond the linear region of the standard curve were diluted with PBS and reassayed.

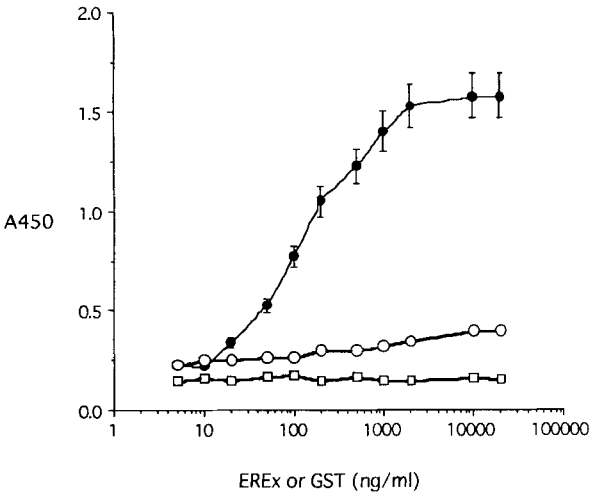

Fig. 1. Standard curve for soluble EpoR ELISA. ELISA was performed as described in Materials and Methods. EREx (solid circles) or GST (open squares) were added at the indicated concentration, and the absorbance at 450 nm was determined. In a separate experiment, preimmune sheep IgG was used to coat the wells rather than sheep anti-EREx, followed by the indicated concentrations of EREx (open circles). Biotinylated anti-EREx was used as a last step for all three curves. Quadruplicate determinations were done for each point. Error bars indicate ±SE of measurement.

Preparation of Plasma and Serum for ELISA

Blood was collected from normal volunteers, and both plasma and serum were prepared. EDTA anticoagulant was used when preparing plasma. Samples were centrifuged at 15,000 rpm in a microcentrifuge, and plasma or serum were removed and frozen at −20°C until the assay was performed. Prolonged freezing of plasma or serum, or storage at room temperature for 12 hr, had no effect on the results obtained (data not shown). Spun hematocrits were determined for all normal subjects. Blood was obtained following informed consent, as approved by our institutional review board.

For some experiments, plasma was depleted of IgG by adsorption with protein A agarose (25 μl of packed beads per ml of plasma) for 1 hr at 37°C. Plasma was depleted of soluble EpoR by incubation with 10 μg/ml of anti-EREx for 1 hr at 37°C, followed by adsorption with protein G agarose (25 μl of packed beads per ml of plasma). Plasma was ultracentrifuged to remove cellular debris and microparticles in a Beckman preparative ultracentrifuge with an SW41 rotor at 75,000*g* for 45 min (Beckman, Palo Alto, CA). Hypotonic lysis of red cells was performed by incubation of whole blood with an equal volume of water for 1 hr at 4°C, followed by centrifugation to remove unlysed cells.

Immunoblots

One hundred μl of plasma or purified EREx (100 μg/ml) were electrophoresed on 10% SDS gels and transferred to nitrocellulose paper. The blot was developed with affinity-purified anti-EREx, followed by rabbit anti-sheep IgG conjugated to HRP. Bands were visualized using enzyme-linked chemiluminescence.

RESULTS

In order to determine if a soluble form of EpoR exists in human blood, we developed an immunoassay using polyclonal antibodies raised against human EpoR. Sheep were immunized with the recombinant human EpoR protein, EREx. EREx is a fusion protein consisting of glutathione-S-transferase (GST) and the extracellular domain of the EpoR [7]. Affinity-purified antibodies were prepared from the immune serum.

The assay is a two-stage sandwich antigen-capture ELISA using biotinylated anti-EREx. Figure 1 shows an ELISA standard curve constructed with purified EREx (solid circles). The assay is linear between 10–2,000 ng/ml of EREx. As described in Materials and Methods, the anti-EREx is extensively adsorbed to remove anti-GST antibodies. This procedure makes the assay insensitive to GST (open squares). When preimmune IgG is substituted for anti-EREx (open circles), little signal is generated, indicating the specificity of the assay.

We tested normal human plasma with this assay. As demonstrated in Table I, some individuals have a component in plasma which reacts with this antibody (see value for Normal 1), and some individuals do not (normal 2). Protein A adsorption of the plasma with high amounts

TABLE I. Specificity of Soluble EpoR ELISA in Plasma*

	Normal 1	Normal 2
Plasma	1,250 ng/ml	<10 ng/ml
Protein A adsorption of plasma	1,350 ng/ml	
Adsorption of plasma with anti-EREx	125 ng/ml	
Adsorption of anti-EREx with EREx	100 ng/ml	
Ultracentrifugation of plasma	1,300 ng/ml	
Hemolysis of blood prior to preparation of plasma		<10 ng/ml

*Two normal individuals were tested using the assay. Normal 1 has a high level of soluble EpoR, and normal 2 has a low level of soluble EpoR. Values for sEpoR levels were extrapolated from the EREx standard curve. The plasma was treated as described in Materials and Methods in order to test for the specificity of the assay. Quadruplicate determinations were done for each point. Standard errors (not shown) were similar to those in Figure 1.

of soluble EpoR prior to assaying (Table I) had no effect on the amount of signal generated, ruling out an anti-heterophile or anti-idiotype artifact. Immunodepletion of the plasma with affinity-purified anti-EREx prior to assay (Table I), or preincubation of the affinity-purified anti-EREx with EREx agarose prior to use in the assay (Table I), markedly decreased the signal, indicating the specificity of the measurement in plasma. Omission of any of the immunochemical reagents also eliminated the signal (data not shown). Ultracentrifugation of the plasma had no effect on the level of soluble EpoR detected (Table I). Hypotonic hemolysis of red cells was performed prior to plasma isolation with the blood from one individual, with no detectable soluble EpoR. There was no difference in the low value before or after hemolysis (Table I), indicating that the detected protein is not an artifact due to hemolysis.

The plasma of 20 normal subjects was tested in the ELISA (10 males and 10 females, age range, 20–40 years). Results are shown in Figure 2. There was a wide variation in amount of signal obtained among normal individuals. Six normals had levels below the detection limit of 10 ng/ml. The 14 with detectable levels had between 35–2,200 ng/ml. The average value obtained for these 20 normal subjects was 550 ± 735 ng/ml (a value <10 ng/ml was considered to be zero in determining this average).

When the assay was done with serum, much lower values were obtained (Fig. 2). Every individual with a measurable level of soluble EpoR had a decrease in signal when serum was compared with plasma. Several individuals went from detectable to undetectable levels of soluble EpoR following clot formation. For those that had a measurable level in both plasma and serum, the decrease ranged from 50% to >99%, with an average decrease of 88%. As shown in Figure 3, there was no correlation between spun hematocrit and amount of soluble Epo receptor in the 14 normal subjects with detectable plasma-soluble EpoR ($r = -0.17$).

In order to determine the molecular weight of the EpoR protein detected in plasma, immunoblots were performed

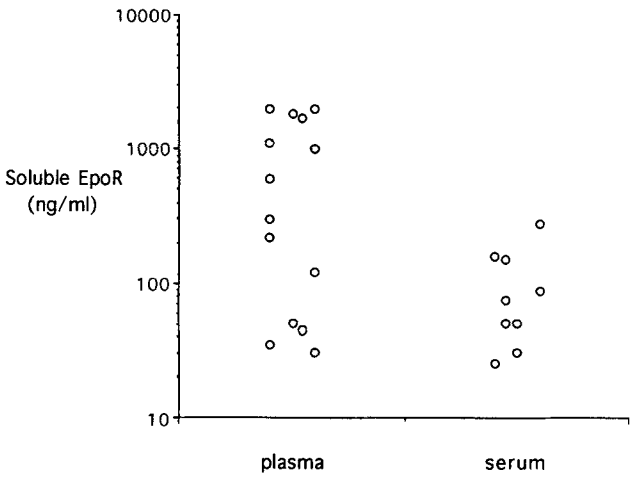

Fig. 2. Soluble EpoR levels in normal plasma and serum. Plasma and serum from 20 normal individuals (10 females and 10 males, age range, 20–40 years) were tested in the soluble EpoR ELISA. Values were extrapolated from the EREx standard curve. Quadruplicate determinations were done for each point. Standard error bars were similar to Figure 1 and have been omitted for clarity. The lower limit of detection of the soluble EpoR ELISA is 10 ng/ml. Measurable levels of soluble EpoR were found in the plasma of 14 normal individuals, and in the serum of 9 normals, and are shown here.

(Fig. 4). Plasma was electrophoresed and then immunoblotted with anti-EpoR antibodies. We were unable to obtain consistent evidence of soluble EpoR with this technique using any of the normal plasmas (data not shown). However, a patient with acute myeloid leukemia and 5 μg/ml of soluble EpoR by ELISA had a 27-kDa protein identified (arrow in Fig. 4, lane 1), which was not seen in a normal person with soluble EpoR level below the ELISA limit of detection (Fig. 4, lane 2). A 66-kDa protein is seen on the immunoblot in both the patient with acute leukemia (Fig. 4, lane 1) and the normal individual without detectable soluble EpoR (Fig. 4, lane 2). This 66-kDa protein probably represents nonspecific interaction of the antibodies with albumin. The presence

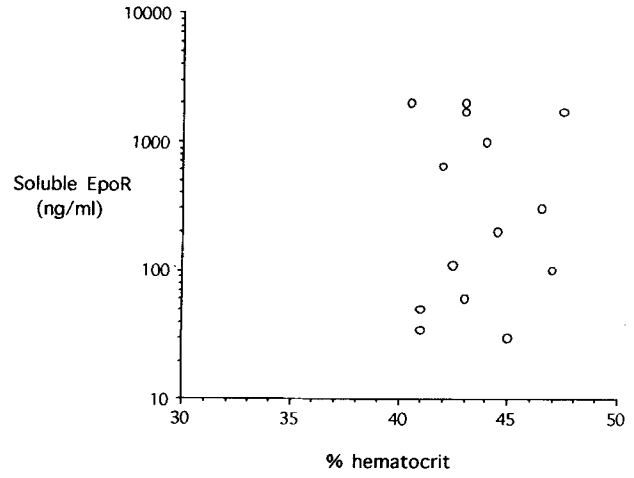

Fig. 3. Effect of hematocrit on the soluble EpoR levels in normal individuals. Soluble EpoR levels in plasma from the 14 normals depicted in Figure 2 are expressed as a function of spun hematocrit. Standard error bars have been omitted for clarity.

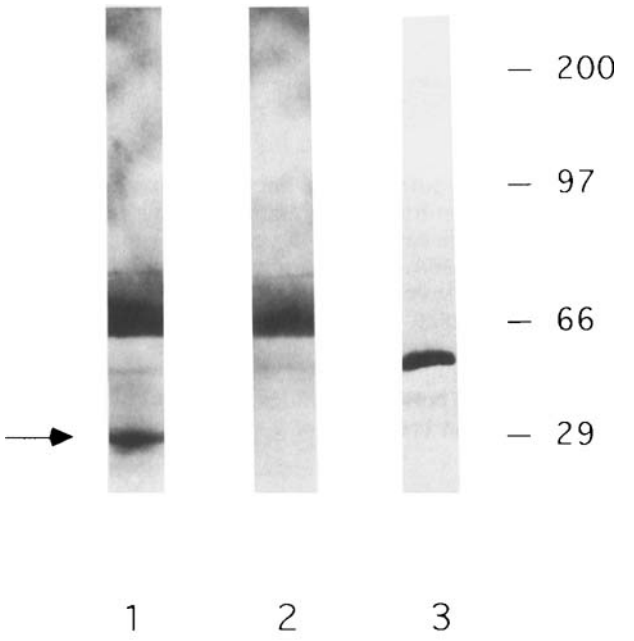

Fig. 4. Immunoblot of the soluble EpoR. Plasma or purified EREx was electrophoresed on 10% SDS gels and then immunoblotted with anti-EREx as described in Methods. Lane 1: plasma from a patient with AML and 5 μg/ml of soluble EpoR. Lane 2: plasma from a normal with an undetectable amount of soluble EpoR. Lane 3: purified EREx. The migration of the soluble EpoR in plasma is indicated by the arrow. The 66 kDa protein seen in lanes 1 and 2 was routinely seen in all plasmas tested as well as with preimmune antibody (data not shown) and probably represents serum albumin. The mobility of molecular weight markers are indicated on the right.

of this crossreacting protein does not influence the ELISA measurements, as this band is routinely seen in normal subjects with no detectable soluble EpoR, as well as in normals with high amounts of soluble EpoR (data not shown). The specificity of the antibody is demonstrated by an immunoblot of purified EREx (Fig. 4, lane 3).

DISCUSSION

In this report we describe an ELISA which detects a potential soluble form of EpoR in human plasma and serum. It is unlikely that the detected substance is artifactual. The signal seen with the assay in individuals with a high value is not due to an anti-heterophile antibody effect because, as shown in Table I, protein A adsorption of plasma has no effect on the value obtained, and adsorption of plasma with anti-EREx, or use of anti-EREx which was preadsorbed with EREx, eliminated the signal. It is also unlikely that an anti-heterophile antibody would show such a large difference between plasma and serum (Fig. 2). The anti-EREx is specific for EpoR, as described in Materials and Methods.

Using this assay, we have determined a surprisingly large variation in normal values for soluble EpoR (Fig. 2). The level of soluble EpoR does not appear to be a function of hematocrit (Fig. 3). It is possible that the level of soluble EpoR is a reflection of the amount of ongoing erythropoiesis in a given individual [14]. If this is so, it will be interesting to compare the amount of soluble EpoR with other measures of erythropoietic activity, such as transferrin receptor levels or Epo levels. If the large range of normal values for soluble EpoR is confirmed in further studies, it also suggests that the process responsible for producing soluble EpoR (be it erythropoiesis or otherwise) varies widely in activity in normal individuals.

The potential plasma EpoR we detected in our ELISA appears to be a true soluble receptor. Ultracentrifugation, which should pellet cell fragments or microvesicles of cells containing full-length EpoR, has no effect on the value obtained (Table I). The molecular weight of the soluble EpoR detected on immunoblot of plasma from an individual with a high level of soluble EpoR (27 kDa) is consistent with that of the EpoR extracellular domain (Fig. 4).

The difference in soluble EpoR levels between plasma and serum is unexpected and unexplained. It is possible that soluble EpoR is consumed by proteolysis during coagulation or adheres to the fibrin clot. If soluble EpoR does have a physiologic function (see below), this finding suggests an intriguing connection between hemostasis and erythropoiesis.

Baynes et al. [14] have recently described the detection of soluble EpoR in human serum by immunoblot. They find an increase in soluble EpoR levels under conditions of increased erythropoiesis. Their results differ from the

data presented here in several regards. We have found that most normal individuals have a detectable level of soluble EpoR in serum or plasma, while Baynes et al. [14] report that most normals do not have detectable amounts in serum. This discrepancy may result from the fact that serum contains significantly less soluble EpoR, as compared to plasma (Fig. 2). The ELISA described here may also be more sensitive than an immunoblot. One obvious advantage of an ELISA compared to an immunoblot is improved quantitation.

The molecular weight of the soluble EpoR detected in our study (27 kDa, see Fig. 4) differs significantly from that in the study of Baynes et al. [14] (34 kDa). The reasons for this are unclear. It is possible that the soluble EpoR from our patient with acute leukemia is abnormally glycosylated or has some other structural alteration, compared with normal soluble EpoR. The explanation for this difference will await the structural characterization of soluble EpoR from blood.

Proof of the existence of a soluble EpoR in human plasma or serum will require its isolation and structural analysis. Several functions for a soluble EpoR may be imagined, including either an inhibitor or carrier for Epo. We have found that a recombinant EpoR fusion protein containing the alternatively spliced soluble EpoR sequence (obtained from the cDNA data) does not bind Epo [15]. The possibility that the EpoR dimerizes [9,10] further expands the potential role for this soluble receptor. If the soluble EpoR level reflects erythropoiesis as suggested by Baynes et al, the assay described here could have clinical utility.

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